CROATICA CHEMICA ACTA
CCACAA **78** (3) 367–374 (2005)

ISSN-0011-1643
CCA-3022
Original Scientific Paper

Flavonoids as Inhibitors of Lck and Fyn Kinases*

Maja Čalić, Dubravko Jelić, Roberto Antolović, Krunoslav Nujić, Nikola Marjanović, Darija Stupin Polančec, Smiljka Vikić-Topić, and Donatella Verbanac**

PLIVA – Research Institute Ltd, Prilaz Baruna Filipovića 29, HR-10000, Zagreb, Croatia

RECEIVED NOVEMBER 11, 2004; REVISED MARCH 31, 2005; ACCEPTED APRIL 4, 2005

Phosphorylation of tyrosine residues constitutes a unique signaling pathway involved in regulation of most cellular processes responding to different extracellular stimuli. The enzymes that carry out this modification are tyrosine kinases. These enzymes enable the transfer of γ-phosphate from ATP to the phenol –OH group of tyrosine on protein substrates. Development of specific and potent protein kinase inhibitors is important not only for treatment of diseases, but also as a tool to investigate the physiological roles of protein kinases. Flavonoids are biologically active polyphenol compounds naturally occurring in many plants. They are recognized as inhibitors of Fyn and Lck protein kinases, two representatives of the *Src* family of non-receptor kinases involved in T-cell signaling transport. In the described experiments, the inhibitory activity of flavonoids on Fyn and Lck kinases was monitored by the ELISA method. Myricetin showed the highest inhibitory effect, and no ATP-competitive mechanism of inhibition was observed on Fyn tyrosine kinase. The affinity of human Fyn and Lck for two different substrates, polypeptide polymer Poly Glu:Tyr (4:1) and peptide M3-01, was also tested.

Keywords
tyrosine kinases
Lck
Fyn
flavonoids
ELISA

INTRODUCTION

As a result of the successful Human Genome Project, the beginning of the 21st century has brought up a new perspective on drug discovery.[1] Gene identification has provided a new paradigm for understanding human diseases at their most fundamental level and defined novel molecular drug targets for the prevention and treatment of diseases.[2] Research activities of many pharmaceutical companies are focused on the protein tyrosine kinases (TKs), a subfamily of the largest known gene family – kinases. It is estimated that the human genome contains more than 2000 different kinases.[3,4]

Protein phosphorylation constitutes a unique mechanism of communication within the cell and between cells in a multicellular organism as a response to different extracellular signals.[5,6,7,8,9] Stimulated by extracellular signals, kinases catalyze transfer of ATP γ-phosphate to a specific amino acid residue on protein substrates.[5,7] Depending on the amino acid residue to be phosphorylated, kinases can be divided into groups such as tyrosine kinases, serine-threonine kinases and histidine kinases.[10,11]

---

* Dedicated to Professor Željko Kućan on the occasion of his 70th birthday. Presented at the Congress of the Croatian Society of Biochemistry and Molecular Biology, $HDBMB_{2004}$, Bjelolasica, Croatia, September 30 – October 2, 2004.

** Author to whom correspondence should be addressed. (E-mail: dubravko.jelic@pliva.hr)

Other enzymes, known as phosphatases, conduct dephosphorylation, a reaction that is also an essential segment of signaling pathways.[6,9] TKs play an important role in different cellular processes (pathways) such as embryogenesis, cell differentiation, growth, metabolism, proliferation and apoptosis.[5,6,7,8,10] Protein phosphorylation by these enzymes is the key element for normal regulation of the mentioned processes.[3,4,7,8] Therefore, irregular activity of TKs could be a cause or a consequence of many diseases such as allergies, immune disorders and carcinomas.[3,7] The possibility of confronting such illnesses with efficient kinase inhibitors is the reason why the drug development of many pharmaceutical companies has been directed in this way.[3,12] The great success achieved by two kinase inhibitor drugs, Herceptin (1998) and Gleevec (2002), has given new optimism to the future projects.[3,4,13] However, the development of efficient protein kinase inhibitors is important not just for treatment of diseases, but also as a tool to investigate the physiological roles of protein kinases.

Two classes of TKs are present in the cell: the transmembrane receptor TKs and the nonreceptor TKs.[7] The nonreceptor tyrosine kinases (TKs) are cytoplasmic proteins, elements of signaling pathways triggered by transmembrane receptor TKs or other cell surface receptors.[7] The largest subfamily of nonreceptor TKs, of nine members, is the *Src* family. *Src* family members participate in a variety of signaling processes, including mitogenesis, T- and B-cell activation and cytoskeleton reconstruction.[7,14,15] Members of this family have also been implicated in several human carcinomas, including breast, lung, and colon cancer.[7] By interrupting or attenuating the signaling processes, we might prevent cell activation, differentiation and growth.

Macromolecular structure of TKs is combined of two domains – a structurally preserved catalytic domain and an evolutionary less preserved regulatory domain.[5,16] ATP binding site is located in the pocket between N- and C-terminal regions in the catalytic domain. Substrates of protein kinase are attached to less known surface depressions, and not to the well-defined ATP binding site. This difference of binding sites in the catalytic domain suggests that remote specific motives are responsible for efficient phosphorylation.[15,16,17]

In our experiments, enzymatic activity of Lck and Fyn kinases in the presence of selected flavonoid compounds using the ELISA method was monitored.[5,18,19,20] Lck and Fyn are two nonreceptor TKs that belong to the previously described *Src* kinase family.[5] They are important components of the regulation of T-cells of the immune system.[21,22] T-cell activation is initiated by antigen recognition by the T-cell receptor (TCR). The TCR contains multiple subunits and interacts with several factors to transduce antigen-stimulated T-cell activation. One of the key steps that initiate TCR activation is the tyrosine phosphorylation of TCR subunits by the *Src* family protein kinases Lck and Fyn. The T-cell receptor phosphorylated by Lck and Fyn recruits the ZAP-70 protein kinase to the receptor complex, which becomes activated and thereafter stimulates downstream signaling pathways. As a result, numerous metabolic changes occur, some of them being the accumulation of Ca^{2+}, phosphatidylinositol breakdown, lymphokine secretion, IL-2 synthesis, and finally T-lymphocyte proliferation.[17,21,22,23]

Flavonoids belong to a large family of polyphenole compounds naturally occurring in many plants.[24] They play an important role in plant growth, development and defense, and even represent the pigments responsible for the yellow, orange or red shades of flowers.[24] According to their basic chemical structure, flavonoids can be divided in many subclasses: chalcones, flavanones, dihydroflavonols, flavan-3-ols, flavones, flavonols, anthocyanidins, isoflavones, neoflavones, *etc*.[25] The structure of some subclasses is presented in Figure 1. More than 8000 flavonoid compounds have been discovered so far, and their number is constantly increasing due to the possible synthesis and modification of these compounds by adding hydroxy, methyl, acyl groups, sugars or other substituents to different C-atoms of two aromatic rings (rings A, B in Table III).[24,26]

Many flavonoids are known for their anti-inflammatory, antiallergic, anticancer, cardioprotective and immunomodulatory activities.[24] Some of these biological activities are assumed to be connected with the antioxidant properties of these compounds, which are expressed by limiting the production of reactive oxygen species and/or their scavenging.[24,25] Flavonoids have been shown to inhibit several enzymes, including hypoxygenases and protein kinases.[24] Many of these compounds are compo-

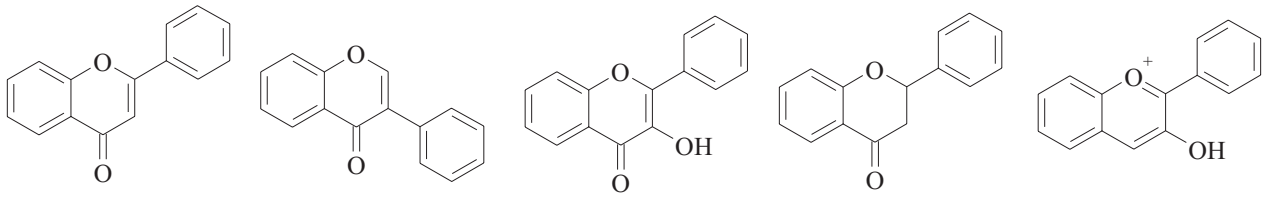


Figure 1. Chemical structure of some flavonoid subclasses.[25]

TABLE I. Flavonoids in food[27]

Food	Compound	Subclass	Amount (mg / 100 g)
Cherries (sweet, raw)	Pelargonidin	Anthocyanidin	0.8
Chocolate (dark)	Catechin	Flavan-3-ols	12
	Epicatechin		41.2
Tea leaves (black, dry)	Catechin	Flavan-3-ols	157
	Epicatechin		293.3
Wine (red)	Malvidin	Anthocyanidin	4.2
	Catechin	Flavan-3-ol	8.9
Grapefruit (raw)	Naringenin	Flavanone	78.1
Celery (raw)	Apigenin	Flavone	6.1
	Quercetin	Flavonol	3.5
Cranberry (raw)	Quercetin	Flavonol	14
	Myricetin	Flavonol	4.3
Garlic (raw)	Quercetin	Flavonol	22.6
Orange (raw)	Hesperetin	Flavanone	39
Orange juice (raw)	Hesperetin	Flavanone	13.9
Kale (raw)	Kaempferol	Flavonol	14.6

nents of the everyday human diet. They are present in fruits, vegetables, chocolates, herbs and beverages, such as wine, tea or beer.[24,27,28] Examples of flavonoids in foods and herbs are listed in Tables I and II. It has been reported that the intake of flavonoids with our everyday diet is about 20 mg to 1 g.[29] Food combined with the mentioned biological properties, the so called functional food, has stimulated even greater interest in flavonoid compounds. However, as natural products, flavonoids can vary in amount and structure as a result of genetic factors, climate, soil quality, and other external factors, as well as the type of food processing.[24,28] Therefore, controlled cultivation and subsequent adequate processing are essential to ensure the most consistent concentration of specific flavonoid compounds. Healing effects of many herbs, used in traditional and modern medicine, are also connected with the presence of flavonoids.[24,30]

EXPERIMENTAL

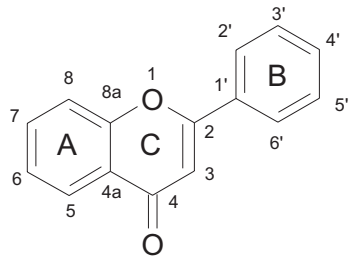
Inhibitory activity of flavonoid compounds on Fyn and Lck was monitored by the ELISA method, described as follows. The same method with addition of different ATP concentrations was used to examine the possible ATP-competitive mechanism of inhibition of flavonoids on Fyn and Lck kinases. Two peptides were used as tyrosine kinase substrates – the random polypeptide polymer Poly Glu:Tyr (4:1) (Sigma, P-0275) and M3-01 pentadecapeptide (Eötvos Lorand University, PEP M3-01), with the following amino acid composition: KVEKIGEGTYGVVYK. Compounds to be tested were selected from PLIVA's Compound Library. All selected flavonoid compounds (Table III), as well as staurosporine (Sigma, S-4400), are commercially available (Sigma, InterBioScreen, Alexis, *etc*.). Protein lymphocyte kinases, Lck and Fyn, were expressed in the Sf9 baculovirus expression system, isolated, purified and characterized using literature protocols,[31,32,33] slightly modified according to internal capabilities. BSA was obtained from Sigma (A-2153) and peroxidase-labeled anti-phosphotyrosine antibody from Calbiochem (525320).

Experiments were conducted in 96-well Dynex Immulon 2 HB microtiter plates (flat bottom, transparent). Substrates were diluted (0.1 mg/ml) in coating buffer (0.1 M $NaHCO_3/Na_2CO_3$, pH = 9.6), and added to wells in a 100 µl volume to adsorb overnight at 37 °C, and kept in a dark and dry place. At the start of the experiment, plates were washed with TBS-T (3x, 200 µl/well, 5–7 min). 47 µl of 2×10^{-6} M ATP was then added to each well. Selected flavonoid compounds were diluted in 100 % DMSO. Stock solutions in concentrations from 10^{-2} M to 10^{-9} M were made and added to wells in a volume of 3 µl. Each compound was added to a different column of the microtiter plate in a decreasing concentration range. Lck and Fyn kinases were diluted in kinase buffer (25 mmol dm^{-3} hepes, 10 mmol dm^{-3} $MnCl_2$, 10 mmol dm^{-3} $MgCl_2$, 0.2 mmol dm^{-3} DTT, pH = 7.4) and added to wells, 50 µl/well. Kinase reaction lasted 20 min, the content of plates was then discarded. Plates were washed with TBS-T (3x, 200 µl/well, 5–7 min). A 2.5 % dilution of BSA in TBS-T was added to

TABLE II. Flavonoids in herbs[24,30]

Herb	Active flavonoid component	Use
Calendula flowers *(Calendula officinalis)*	Isoquercitrin, rutin, narcissin	Sun burns Gum inflammation Wound healing Immunomodulatoryproperties
Chamomile flowers *(Matricaria chamomilla)*	Apigenin and luteolin glycosides	Gastrointestinal complaints Skin and mucosa irritation Gum inflammation Insomnia
Ginkgo leaf extract *(Ginkgo biloba)*	Flavonol glycosides	Alzheimer disease Headache (effects on blood circulation in brain)
Licorice root *(Glycyrrhiza glabra)*	*Glycyrrhizin*-glycosides, flavanones, chalcones, isoflavones	Dyspeptic problems

TABLE III. Tested flavonoid compounds



Subclass	Compound	PLIVA code	Structure
Flavones	Apigenin	PL 29841	5,7,4'-OH
	Acacetin	PL 33652	5,7-OH-4'-OCH_3
	Baicalein	PL 29343	5,6,7-OH
Flavonols	Fisetin	PL 29286	3,7,3',4'-OH
	Gossipin	PL 10327	3,5,7,3',4'-OH-8-OGlc
	Morin	PL 33653	3,5,7,2',4'-OH
	Myricetin	PL 33959	3,5,7,3',4',5'-OH
	Quercetin	PL 33551	3,5,7,3',4'-OH
Anthocyanidins	Pelargonidine	PL 33952	3,5,7,4'-OH-1-Cl^-

wells, 100 µl/well, and left to react (blocking reaction) for 30 min, then the content of plates was discarded. 100 µl/well of peroxidase-labeled anti-phospho-tyrosine antibody dilution in 2.5 % BSA was then added, in a concentration of 1:6000. Reaction lasted for 40 min and the content of plates was discarded. Plates were washed with TBS-T (3x, 200 µl/well, 5–7 min) and TBS (1x, 200 µl/well, 5–7 min). 100 µl/well of peroxidase detection system was added: 12.5 ml citric acid, 12.5 ml Na_2HPO_4, 20 mg phenylenediamine (Sigma, P-8287), 20 µl H_2O_2 and MilliQ H_2O to a final volume of 50 µl. After 10–30 minutes, the reaction was stopped with 25 µl of 1.25 M H_2SO_4. The developed color intensity was measured spectrophotometrically at 490 nm. IC_{50} was determined using the GraphPad Prism software, v. 3.02.

Possible ATP-competitive mechanism of inhibition of flavonoids on Fyn kinase was assayed using a specific compound – myricetin. This most potent inhibitor among all tested compounds was assayed for the mechanism of action in the presence of different ATP concentrations (1, 10 and 100 µmol dm^{-3} as final). The substrate used was Poly Glu:Tyr (4:1). Staurosporine, an ATP-competitive kinase inhibitor, was used as a control.

RESULTS

Inhibitory activity of flavonoid compounds on Fyn and Lck kinases was expressed as IC_{50}, which is defined as the compound concentration for 50 % inhibition of kinase. From experimental data, IC_{50} was determined by charting the logarithm of concentration of the tested flavonoid compound in dependence on A_{490}, and IC_{50} represented the point of inflection on the obtained curve.

TABLE IV. Inhibitory activity of flavonoid compounds on Fyn and Lck kinases; substrates: Poly Glu:Tyr (4:1), M3-01 peptide

PL	Compound	IC_{50} (Fyn) / µmol dm^{-3}		IC_{50} (Lck) / µmol dm^{-3}	
		Poly Glu:Tyr (4:1)	pep M3-01	Poly Glu:Tyr (4:1)	pep M3-01
2638	Staurosporine	0.014	0.015	0.024	0.049
29841	Apigenin	8.6	4.8	14.6	57.5
33652	Gossipin	17.5	29.3	>100	>100
29343	Morin	11.9	15.3	14.4	103
29286	Myricetin	1.1	0.8	1.2	3.4
10327	Quercetin	1.9	1.8	8.3	3.3
33653	Acacetin	20.3	5.8	>100	>100
33959	Baicalein	14.7	13.5	>100	62.0
33551	Fisetin	29.8	8.6	>100	>100
33952	Pelargonidine Cl	28.6	111	>100	>100

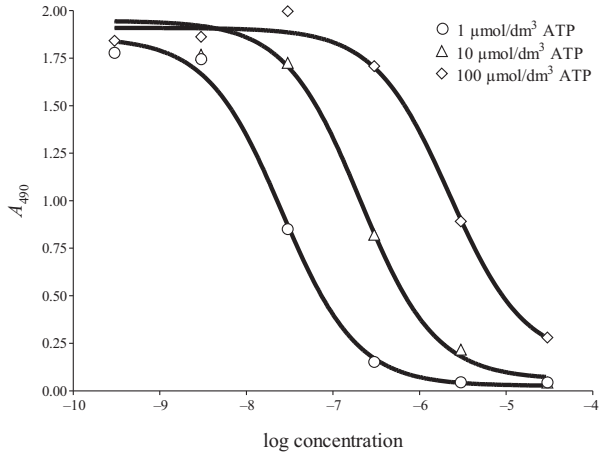


Figure 2. Inhibitory activity of Staurosporine on Fyn kinase in the presence of different concentrations of ATP (1,10 and 100 μmol dm^{-3}). Poly Glu:Tyr (4:1) was used as substrate.

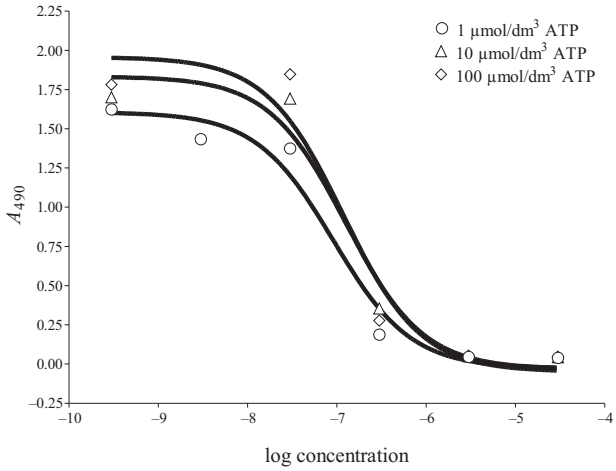


Figure 3. Inhibitory activity of Myricetin on Fyn kinase in the presence of different concentrations of ATP (1,10 and 100 μmol dm^{-3}). Poly Glu:Tyr (4:1) was used as substrate.

TABLE V. Inhibitory activity of myricetin and staurosporine on Fyn kinase in the presence of different concentrations of ATP; substrate, Poly Glu:Tyr (4:1)

	[ATP]/μmol dm^{-3}	1	10	100
PL	Compound	IC_{50}(Fyn) / μmol dm^{-3}		
2638	Staurosporine	0.026	0.21	2.2
29286	Myricetin	0.091	0.13	0.12

Calculations were done using the computer program Graph Pad Prism software, v. 3.02.

Final inhibitory activity results under our experimental conditions (r.t., 0.01 mg of substrate, 1:200 dilution of enzyme, 1 μmol dm^{-3} ATP, 3×10^{-4} to 3×10^{-11} mol dm^{-3} concentrations of selected flavonoid compounds and 3×10^{-5} to 3×10^{-12} mol dm^{-3} concentrations of staurosporine, as final) varied from 0.8 to more than 100 μmol dm^{-3}, depending on the flavonoid and/or enzyme used. While examining the possible ATP-competitive mechanism of inhibition of flavonoid compounds, IC_{50} for myricetin varied very little from 0.09–0.13 μmol dm^{-3} in the presence of different ATP concentrations (all other experimental conditions were the same as previously described). In the case of staurosporine, the obtained IC_{50} values were 0.02 μmol dm^{-3} for 1 μmol dm^{-3} ATP, 0.2 μmol dm^{-3} for 10 μmol dm^{-3} ATP and 2.2 μmol dm^{-3} for 100 μmol dm^{-3} ATP.

Final inhibitory activity results of all flavonoid compounds are given in Tables IV and V, and in Figures 2 and 3 for ATP-competition.

DISCUSSION

The aim of this work was to obtain the structure and activity relationship of the tested compounds for selected kinases. From the results given in Table IV, we can see that the tested flavonoid compounds have shown higher inhibitory activity towards Fyn tyrosine kinase compared to Lck. Depending on the functional groups present on three different flavonoid rings, A, B and C (Table III), their IC_{50} varied from 0.8 μmol dm^{-3} to more than 100 μmol dm^{-3}. The best results for IC_{50} on both Fyn and Lck kinases were shown by myricetin and quercetin (Table IV). Other tested compounds showed inhibition only on Fyn kinase (with the exception of apigenin and morin, which showed some weak inhibitory activity also on Lck). Although only activities of myricetin and quercetin are in a low micromolar range for both kinases, it is evident that flavonoids as a class exert inhibitory activity on these two tyrosine kinases. Certain changes in functional groups on the flavonoid scaffold could be responsible for the decrease of inhibitory activity, as can be seen in examples of pelargonidine or fisetin (Table IV, different distribution of –OH groups around the flavonoid scaffold). Flavonols are generally more active than flavones and anthocyanidines (Table III). Decrease of activity of flavonols is observed when some sterically significant group is linked to the scaffold, as can be seen in the gossypin example with the sugar moiety linked to position 8 of flavonole A-ring (Table III). It is hard to predict the physiological or pharmacological relevance of the inhibition potential of flavonoids shown in this work without more extensive *in vivo* experiments. Based on *in vitro* knowledge from this work, it is obvious that some limited synthesis efforts could lead us to more potent, efficient and selective flavonoid compounds. But, it is also necessary to perform some additional experiments of further profiling of the data obtained. Testing the cytotoxicity effect of flavonoids upon T-lymphocytes and other cell lines could be the next step.

As a standard kinase inhibitor, staurosporine has demonstrated nonselective inhibition of both tyrosine kinases in a low nmol dm^{-3} concentration, as expected.

Poly Glu:Tyr (4:1) and M3-01 peptide (KVEKIGEGTYGVVYK) used in these experiments are two structurally very distinctive substrates. Synthesis of M3-01 peptide was performed on our request at Eötvos Lorand University, with the purpose to examine the specificity of this particular substrate toward Lck and Fyn kinases. This high specificity profile was given in the TECAN protocol for quantification of tyrosine kinase activity with fluorescence polarization (the protocol is available and was kindly provided to the authors by TECAN and PanVera/Invitrogen).[34] However, based on the *in vitro* data obtained, no significant difference in kinase affinity for phosphorylation of tyrosine residues on these two substrates has been observed. Since M3-01 peptide has two tyrosine residues, and the random polypeptide Poly Glu:Tyr (4:1) has many of them randomly distributed, it is obvious that the structural difference between the tested substrates is not relevant for kinase affinity toward phosphorylation of tyrosine residues on these substrates.

No ATP-competitive mechanism of inhibition for myricetin on Fyn tyrosine kinase was observed (Table V, Figure 3). A difference in the maximum magnitude of OD signal for three different concentrations of ATP was recorded in the competitiveness experiment for myricetin (Figure 3, maximum OD is 1.6 for 1 μmol dm^{-3}, 1.8 for 10 μmol dm^{-3} and 1.9 for 100 μmol dm^{-3} ATP concentration). The reason lies in the fact that these three ATP: compound ratios were in three different plates and the color intensity developed in the assay was different for these 3 plates. But, IC_{50} values were unique afterwards, irrelevant for the maximum OD developed (if not more than 2). This competitiveness result indicates that myricetin inhibits Fyn by binding on a different site, which is not the alosteric-ATP binding pocket. It is known that Lck and Fyn participate in phosphotyrosine-dependent protein-protein interactions through the *Src* homology-2 (SH2) modular binding unit domain.[5,10] Most probably, this domain (SH2) could be the site where the inhibition by myricetin occurs. ATP-competitive inhibition mechanism was obtained and confirmed in the case of the general tyrosine kinase inhibitor – staurosporine (Table V, Figure 2).

Although flavonoids show positive biological activities, there is evidence that higher concentrations of some flavonoids may act as cytotoxic agents, pro-oxidants (generation of free radicals), inducers of apoptosis, mutagens, and inhibitors of key enzymes involved in hormone metabolism.[29,35] Flavonoids are also involved in plant defense against microorganisms.[24,36] Antibacterial activity, if detected, could open a new door to the possible application of herein tested flavonoid compounds. Natural flavonoid preparations are mixtures of different flavonoid compounds.[24,37] Some experiments indicate that the synergistic effect generated in this way shows greater activity than flavonoids alone as genuine compounds.[37] This also means that, perhaps, different results could be obtained if the flavonoid mixture were tested on Lck and Fyn kinases, especially *in vivo*. Flavonoids may interact with the absorption of other drugs, generating either positive or negative outcomes.[26] This could also be a useful guideline for the future drug development experiments.

CONCLUSIONS

– Flavonoid compounds have shown inhibitory activity towards Fyn and Lck tyrosine kinases. Fyn kinase has been found to be more sensitive to flavonoid inhibition than Lck.

– No significant difference in kinase affinity toward phosphorylation of tyrosine residues on two structurally diverse substrates, Poly Glu:Tyr (4:1) and M3-01 peptide (KVEKIGEGTYGVVYK), has been observed. Therefore, even by using different substrates in this method, the obtained values for the flavonoids inhibition of Fyn and Lck are accurate and reliable.

– No ATP-competitive mechanism of inhibition of myricetin has been observed on Fyn tyrosine kinase. This indicates that presumably myricetin inhibits Fyn by binding on a different site of the protein, distinctive from the ATP-binding site. Since all tested compounds are members of the flavonoid class of compounds, we can conclude that the mechanism of inhibition might be the same for all analogues.

– ATP-competitive mechanism of inhibition has been obtained in the case of staurosporine – a standard inhibitor of many protein kinases.

Acknowledgements. – The authors are grateful to all persons participating in Lck and Fyn kinases expression, isolation and characterization. Also, we appreciate the great help we received from our technicians Ana Mokrović, Klara Markušić, Andrej Tkalčević and Željka Tolić.

REFERENCES

1. J. C. Venter, M. D. Adams, E. W. Myers, P. W. Li, R. J. Mural, G. G. Sutton, H. O. Smith, M. Yandell, C. A. Evans, R. A. Holt, J. D. Gocayne, P. Amanatides, R. M. Ballew, D. H. Huson, J. R. Wortman, Q. Zhang, C. D. Kodira, X. H. Zheng, L. Chen, M. Skupski, G. Subramanian, P. D. Thomas, J. Zhang, Gabor G. L. Miklos, C. Nelson, S. Broder, A. G. Clark, J. Nadeau, V. A. McKusick, N. Zinder, A. J. Levine, R. J. Roberts, M. Simon, C. Slayman, M. Hunkapiller, R. Bolanos, A. Delcher, I. Dew, D. Fasulo, M. Flanigan, L. Florea, A. Halpern, S. Hannenhalli, S. Kravitz, S. Levy, C. Mobarry, K. Reinert, K. Remington, J. Abu-Threideh, E. Beasley, K. Biddick, V. Bonazzi, R. Brandon, M. Cargill, I. Chandramouliswaran, R. Charlab, K. Chaturvedi, Z. Deng, V. Di Francesco, P. Dunn, K. Eilbeck, C. Evangelista, A. E.

Gabrielian, W. Gan, W. Ge, F. Gong, Z. Gu, P. Guan, T. J. Heiman, M. E. Higgins, R. R. Ji, Z. Ke, K. A. Ketchum, Z. Lai, Y. Lei, Z. Li, J. Li, Y. Liang, X. Lin, F. Lu, G. V. Merkulov, N. Milshina, H. M. Moore, A. K. Naik, V. A. Narayan, B. Neelam, D. Nusskern, D. B. Rusch, S. Salzberg, W. Shao, B. Shue, J. Sun, Z. Wang, A. Wang, X. Wang, J. Wang, M. Wei, R. Wides, C. Xiao, C. Yan, A. Yao, J. Ye, M. Zhan, W. Zhang, H. Zhang, Q. Zhao, L Zheng, F. Zhong, W. Zhong, S. Zhu, S. Zhao, D. Gilbert, S. Baumhueter, G. Spier, C. Carter, A. Cravchik, T. Woodage, F. Ali, H. An, A. Awe, D. Baldwin, H. Baden, M. Barnstead, I. Barrow, K. Beeson, D. Busam, A. Carver, A. Center, M. L. Cheng, L. Curry, S. Danaher, L. Davenport, R. Desilets, S. Dietz, K. Dodson, L. Doup, S. Ferriera, N. Garg, A. Gluecksmann, B. Hart, J. Haynes, C. Haynes, C. Heiner, S. Hladun, D Hostin, J. Houck, T. Howland, C. Ibegwam, J. Johnson, F. Kalush, L. Kline, S. Koduru, A. Love, F. Mann, D. May, S. McCawley, T. McIntosh, I. McMullen, M. Moy, L. Moy, B. Murphy, K. Nelson, C. Pfannkoch, E. Pratts, V. Puri, H. Qureshi, M. Reardon, R. Rodriguez, Y. H. Rogers, D. Romblad, B. Ruhfel, R. Scott, C. Sitter, M. Smallwood, E. Stewart, R. Strong, E. Suh, R. Thomas, N. N. Tint, S. Tse, C. Vech, G. Wang, J. Wetter, S. Williams, M. Williams, S. Windsor, E. Winn-Deen, K. Wolfe, J. Zaveri, K. Zaveri, J. F. Abril, R. Guigo, M. J. Campbell, K. V. Sjolander, B. Karlak, A. Kejariwal, H. Mi, B. Lazareva, T. Hatton, A. Narechania, K. Diemer, A. Muruganujan, N. Guo, S. Sato, V. Bafna, S. Istrail, R. Lippert, R. Schwartz, B. Walenz, S. Yooseph, D. Allen, A. Basu, J. Baxendale, L. Blick, M. Caminha, J. Carnes-Stine, P. Caulk, Y. H. Chiang, M. Coyne, C. Dahlke, A. Mays, M. Dombroski, M. Donnelly, D. Ely, S. Esparham, C. Fosler, H. Gire, S. Glanowski, K. Glasser, A. Glodek, M. Gorokhov, K. Graham, B. Gropman, M. Harris, J. Heil, S. Henderson, J. Hoover, D. Jennings, C. Jordan, J. Jordan, J. Kasha, L. Kagan, C. Kraft, A. Levitsky, M. Lewis, X. Liu, J. Lopez, D. Ma, W. Majoros, J. McDaniel, S. Murphy, M. Newman, T. Nguyen, N. Nguyen, M. Nodell, S. Pan, J. Peck, M. Peterson, W. Rowe, R. Sanders, J. Scott, M. Simpson, T. Smith, A. Sprague, T. Stockwell, R. Turner, E. Venter, M. Wang, M. Wen, D. Wu, M. Wu, A. Xia, A. Zandieh, and X. Zhu, *Science* **291** (2001) 1304–1351.

2. E. H. Ohlstein, R. R. Ruffolo Jr., and J. D. Elliott, *Annu. Rev. Pharmacol. Toxicol.* **40** (2000) 177–191.
3. A. J. Bridges, *Emerging Drugs* **3** (1998) 279–292.
4. P. Choen, *Nature Reviews* **1** (2002) 309–315.
5. S. K. Hanks and T. Hunter, *FASEB J.* **9** (1995) 576–596.
6. L. N. Johnson and R. J. Lewis, *Chem. Rev.* **101** (2001) 2209–2242.
7. S. R. Hubbard, and J. H. Till, *Annu. Rev. Biochem.* **69** (2000) 373–398.
8. T. Hunter, *Cell* **80** (1995) 225–236.
9. B. G. Neel and N. K. Tonks, *Curr. Opin. Cell. Biol.* **9** (1997) 193–204.
10. D. Jelić and W. Brandt, *8th Summer School on Biophysics*, Rovinj, Croatia, September 14–26, 2003, P-105.
11. H. Saito, *Chem. Rev.* **101** (2001) 2497–2510.
12. S. S. Taylor and E. Radzio-Andzelm, *Curr. Opin. Chem. Biol.* **1** (1997) 219–226.
13. M. Elis, *Oncologist.* **9** (2004) 20–26.
14. H. M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T. N. Bhat, H. Weissig, I. N. Shindyalov, and P. E. Bourne, *Nucl. Acids Res.* **28** (2000) 235–242.
15. J. A. Adams, *Chem. Rev.* **101** (2001) 2271–2290.
16. G. Scapin, *Drug Discovery Today* **7** (2002) 601–611.
17. M. Shih, *Lck and Fyn tyrosine kinases in initiation of TCR activation*, http://www.biocarta.com/pathfiles/h_tcraPathway.asp (20. 05. 2004).
18. J. R. Crowther, *ELISA: Theory and practice*, Methods in Molecular Biology, Vol. 42, 1st ed., Humana Press Inc., Totowa, NJ, 1995.
19. Y. Abe, T. Sagawa, K. Sakai, and S. Kimura, *Clin. Chim. Acta* **168** (1987) 87–95.
20. P. E. Anderoti, G. V. Ludwig, A. H. Peruski, J. J. Tuite, S. S. Morse, and L. F. Peruski Jr., *BioTechniques* **35** (2003) 850–859.
21. K. Suzuki-Inoue, D. Tulasne, Y. Shen, T. Bori-Sanz, O. Inoue, S. M. Jung, M. Moroi, R. K. Andrews, M. C. Berndt, and S. P. Watson, *J. Biol. Chem.* **227** (2002) 21561–21566.
22. Q. Tang, S. K. Subudhi, K. J. Henriksen, C. G. Long, F. Vives, and J. A. Bluestone, *J. Immunol.* **168** (2002) 4480–4487.
23. L. E. Samelson, M. D. Patel, A. M. Weissman, J. B. Harford, and R. D. Klausner, *Cell* **46** (1986) 1083–1090.
24. P. Pietta, C. Gardana, and A. Pietta, *Flavonoids in Herbs,* in: C. Rice-Evans and L. Packer (Eds.), *Flavonoids in Health and Disease*, 2nd ed., Marcel Dekker Inc., New York/Basel, 2003, pp. 43–70.
25. W. Bors, C. Michel and K. Stettmaier, *Flavonoids and their free radical reactions* (2001) http://www.medicine.uiowa.edu/FRRB/VirtualSchool/Bors_Flavonoids.pdf (25. 05.2004).
26. U. Wenzel, S. Kuntz, and H. Daniel, *J. Pharmacol. Exp. Ther.* **299** (2001) 351–357.
27. S. Bhagwat, G. R. Beecher, D. B. Haytowitz, J. M. Holden, S. Gebhardt, J. Dwyer, J. Peterson, and A. Eldridge, *Development of database for flavonoids in foods* (2002) http://www.nal.usda.gov/fnic/foodcomp/Data/Other/NDBC26_Flav.pdf (25. 05. 2004.), USDA-ARS.
28. A. Fleuriet and J. J. Macheix, *Phenolic Acids in Fruit and Vegetables,* in: C. Rice-Evans and L. Packer (Eds.), *Flavonoids in Health and Disease*, 2nd ed., Marcel Dekker Inc., New York/Basel, 2003, pp. 1–42.
29. G. C. Yen, P. D. Duh, H. L. Tsai, and S. L. Huang, *Biosci. Biotechnol. Biochem.* **67** (2003) 1215–1222.
30. J. A. Duke, in: D. Štajduhar (Ed.), *Zelena ljekarna* [*Herbal Remedies*] 1st ed., Marijan tisak, Split, 2002 (in Croat.).
31. J. D. Watts, G. M. Wilson, E. Ettenhadieh, I. Clark-Lewis, C. A. Kubanek, C. R. Astell, J. D. Marth, and R. Aebersold, *J. Biol. Chem.* **267** (1992) 901–907.
32. S. E. Ramer, D. G. Winkler, A. Carrera, T. M. Roberts, and C. T. Walsh, *Proc. Natl. Acad. Sci. USA* **88** (1991) 6254–6258.
33. M. Koegl, R. M. Kypta, M. Bergman, K. Alitalo, and S. A. Courtneidge, *Biochem J.* **302** (1994) 737–744.
34. R. Seethala, *Methods* **22** (2000) 61–70.
35. J. P. E. Spencer, H. Schroeter, and C. A. Rice-Evans, *Cytoprotective and Cytotoxic Effects of Flavonoids,* in: C. Rice-Evans and L. Packer (Eds.), *Flavonoids in Health and Disease*, 2nd ed., Marcel Dekker Inc., New York/Basel, 2003, pp. 309–347.
36. H. Arima and G. Danno, *Biosci. Biotechnol. Biochem.* **66** (2002) 1727–1730.
37. H. Arima, H. Ashida, and G. Danno, *Biosci. Biotechnol. Biochem.* **66** (5) (2002) 1009–1014.

SAŽETAK

Flavonoidi kao inhibitori Lck i Fyn kinaza

Maja Čalić, Dubravko Jelić, Roberto Antolović, Krunoslav Nujić, Nikola Marjanović, Darija Stupin Polančec, Smiljka Vikić-Topić i Donatella Verbanac

Tirozinska fosforilacija predstavlja jedinstveni mehanizam prijenosa signala primljenoga iz okoline do stanične jezgre koja će odgovoriti na podražaj. Velika skupina enzima odgovorna za provođenje ove modifikacije poznata je pod jedinstvenim nazivom tirozinske kinaze. One omogućuju prijenos γ-fosfata ATP-a na –OH skupinu tirozina supstrata. Lck i Fyn su dvije nereceptorske *Src* tirozinske kinaze, koje su važne komponente regulacije aktivnosti T-limfocita. Rezultati ispitivanja flavonoida, biološki aktivnih polifenolnih spojeva prirodno prisutnih u mnogim biljkama, upućuju na njihov inhibitorni učinak na proteinske kinaze Fyn i Lck. Najveću inhibiciju pokazao je miricetin, a pri ispitivanju mehanizma inhibicije Fyn kinaze nije ustanovljena kompeticija s ATP-om. Ispitivanje potencijalne inhibicijske aktivnosti flavonoidnih spojeva prema kinazama Fyn i Lck, provedeno je ELISA metodom. Pri tome je uspoređen afinitet ispitivanih enzima prema supstratima s različitim brojem skupina podložnih fosforilaciji, odnosno prema polipeptidnome polimeru glutaminske kiseline i tirozina Poly Glu:Tyr (4:1) i M3-01 peptidu. Razvoj učinkovitih kinaznih inhibitora važan je ne samo za prevenciju i liječenje mnogih bolesti već i za bolje razumijevanje uloge proteinskih kinaza u organizmu.